EXHIBIT B

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A regulatory system for use in gene transfer

(transcription regulation/gene therapy)

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ABSTRACT We recently have demonstrated that a C-terminal deletion mutant of the human progesterone receptor (hPRB891) fails to bind to progesterone but can bind RU 486 (Mifepristone) and other progesterone antagonists. Most significantly, this mutant receptor activates transcription of a reporter gene containing the progesterone response element in the presence of these antagonists. Taking advantage of this finding and the modular nature of functional domains of steroid receptors, we constructed a chimeric regulator (pGL–VP) by fusing the ligand-binding domain of human progesterone receptor hPRB891 to the yeast transcriptional activator GAL4 DNA-binding domain and the herpes simplex virus protein VP16 activation domain. We demonstrated that this chimeric regulator activates target genes containing the GAL4-binding sites in transient transfection assays in response to RU 486. In addition, this regulatory system has been validated by *ex vivo* transplantation of a stable cell line containing both the regulator and a reporter gene into rats. The dosage of RU 486 used is significantly lower than that required for antagonizing progesterone action. The gene-switch system reported here represents a regulatory system, which could be applicable for gene-transfer studies involving animals, as well as humans, in which the delivered gene(s) can be specifically turned on/off in response to an exogenous compound.

The expression of most eukaryotic genes is intricately controlled in response to metabolic, hormonal, or environmental stimuli. Presently, one of the major concerns facing clinical gene-therapy research is how to regulate the expression of transferred exogenous genes within the human body. Also, current protocols for introducing genes into animals are often unsatisfactory due to the constitutive expression of transferred genes. Constitutive gene expression is not physiological and usually leads to down-regulation of effector systems and/or cellular toxicity. The problem of target-gene regulation could be solved by cotransfer of a distinct regulator gene that would, in turn, govern the expression of the target gene. The desired characteristics of this coupled regulatory system are severalfold. Ideally, (*i*) the regulator should be activated only upon administration of an exogenous chemical (ligand) and terminated when the exogenous stimulus is removed; (*ii*) the ligand should be nontoxic and active upon oral administration; and (*iii*) the gene switch should not activate other endogenous cellular genes.

To develop a gene switch/regulatory system with the above characteristics, we used a human progesterone receptor mutant with a 42-amino acid deletion in its C terminal, which has lost its ability to bind progesterone or other endogenous hormones (1). This mutant (hPRB891), however, still can bind the progesterone antagonist RU 486 (2) and paradoxically activate transcription from a reporter gene. Using this finding and the modular nature of functional domains of steroid receptors (3–5), we constructed chimeric regulators that can activate transcription of a target gene in response to RU 486. We present evidence that this activation happens at a concentration of RU 486 that is significantly lower than that required for its antiprogesterone or antiglucocorticoid activity. We also demonstrate that this gene-switch system works not only in an *in vitro* situation, but most importantly, it regulates gene expression *in vivo* in response to a low dose of RU 486.

MATERIALS AND METHODS

Plasmids. To construct the pGL chimera, the *Bam*HI fragment containing the ligand-binding domain of hPRB891 was isolated from plasmid pT7bhPRB-891 (1) and subsequently digested with *Dra* I. The *Dra* I–*Bam*HI fragment (residues 640–891) was then inserted into a *Pvu* II site of plasmid pABgal$_{94}$ containing the yeast activator GAL4 DNA-binding domain (residues 1–94) (6). To construct pGL–VP, a 360-bp *Sal* I–*Bam*HI fragment was isolated from plasmid pMSV VP16-Δ3′-b58N (from A. Friedman and S. McKnight, Carnegie Institution of Washington) and digested with *Rsa* I to generate a 230-bp fragment containing VP16 activation domain (residues 411–487). This fragment was ligated with a *Bam*HI linker (pdGGGATCCC) and subcloned into the *Sal* I and *Bam*HI sites of pBS-KS(−). The *Sal* I end of the resulting plasmid was then blunt-ended and ligated with a *Bgl* II linker (pdCAGATCTG). The *Bgl* II–*Bam*HI fragment was then subcloned into the *Bgl* II site of plasmid pGL (upstream of the N terminus of the GAL4 DNA-binding domain) creating chimeric regulator pGL–VP. Reporter plasmid p17x4-tk-CAT was originally named p17x4-tk-CAT (ΔH/N) (6) and consists of four copies of the GAL4 consensus binding sequence (CGGAGTACTGTCCTCCG) linked to the thymidine kinase gene (*tk*) promoter and the bacterial chloramphenicol acetyltransferase (CAT) gene. Reporter plasmid p17x4-TATA-CAT was constructed by replacing the *Sal* I–*Bgl* II fragment of the *tk* promoter of p17x4-tk-CAT (6) with a *Sal* I–*Bam*HI fragment of pE1bCAT (7) containing the adenovirus major late *E1B* TATA sequence. To construct pGL–VP(H) for establishment of stable cell lines, the chimeric regulator GL–VP insert (digested with *Kpn* I–*Bam*HI from pGL–VP) was inserted into plasmid pCEP4 (Invitrogen), resulting in plasmid pGL–VP(H) in which the expression of GL–VP is driven by the cytomegalovirus promoter. In addition, this plasmid contains the hygromycin-resistance gene enabling selection by hygromycin-B. To construct reporter plasmid p17x4-tk-TH(N), the *Eco*RI–*Xho* I fragment containing the neomycin-resistance gene was isolated from plasmid pPGKneobpA and was inserted into the *Sac* I site of plasmid p17x4-tk-CAT. The CAT gene (*Xho* I–*Sma* I fragment) in plasmid p17x4-tk-CAT(N) was then replaced with the rat tyrosine hydroxylase (TH) gene, a *Xho* I–*Sma* I fragment isolated from prTH-122 (from K. O'Malley, Wash-

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Abbreviations: TH, tyrosine hydroxylase; CAT, chloramphenicol acetyltransferase.

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ington University), creating reporter plasmid p17x4-tk-TH(N).

Transfection. Transfection of CV1 and FR cells, derived from rat fetal skin cells (American Type Culture Collection, CRL 1213), was essentially as described (8). Approximately 1×10^6 cells were seeded into 100-mm dishes 24 hr before transfection. DNA was added to the cell along with 50 μg of Polybrene using the calcium phosphate (Ca_3PO_4) precipitation method. Hormones (in 80% ethanol) were added after glycerol shock, and cells were harvested after 36–48 hr, and CAT activity was measured using 100 μg of cell extracts.

Generations of Stable Cell Lines and Immunoblot Assay. Stable cell lines were established by cotransfecting regulator plasmid pGL-VP(H) and reporter plasmid 17x4-tk-TH(N) into FR cells. They are selected with G418 (100 μg/ml) (GIBCO/BRL) and hygromycin-B (50 μg/ml) (Boehringer Mannheim). Several stable cell lines were selected on the basis of immunoblot analysis of RU 486-induced TH expression. Cell extracts were prepared by adding 600 μl of lysis buffer [50 mM Hepes, pH 7.0/150 mM NaCl/0.5% Nonidet P-40 containing protease inhibitors aprotinin at 2 μg/ml, leupeptin at 2 μg/ml, and pepstatin A at 1 μg/ml and 2 mM benzamidine·HCl (Sigma)] to the cell culture plates (60 mm). The plates were incubated on ice for 20 min, the cell lysates were harvested, and the supernatants were collected after spinning at 10,000 rpm in an Eppendorf centrifuge (5415) for 10 min. For immunoblot analysis, 5 μl of extracts was separated on a SDS/10% PAGE gel and transferred onto a nylon membrane (Micron Separations, 0.45 μm). The blot was probed with anti-TH monoclonal antibody (Boehringer Mannheim) at a concentration of 0.4 μg/ml [Tris/borate/saline-Tween (0.1%) and 1% nonfat milk]. Enhanced chemiluminescence staining was then done according to the manufacturer's instructions (Amersham).

Ex Vivo Transplantation. About 1.8×10^5 stable FR cells (F1.21) (in 200 μl of Dulbecco's modified Eagle's medium) were transplanted (day 1) into the rhomboid and trapezius muscles (right side) of Sprague–Dawley rats (200 g, Harlan Laboratories, Haslett, MI). Twenty-four hours after transplantation, RU 486 (in a total volume of 150 μl in sesame oil) was given to the rats i.p. once a day at the indicated dosage (5 μg/kg, lanes 5–8 and 50 μg/kg, lanes 9–12) for a 2-day period. Rats in a control group (lanes 1–4) were injected with oil only. On the fourth day, rats were sacrificed, and muscles of the injected site were excised (≈20–50 mg of wet weight) and homogenized at 10 mg (wet wt)/ml in lysis buffer, and supernatants were collected as described previously. In these transplantation experiments, each rat also received an i.p. injection of cyclosporine A (10 mg/kg per day, from day 1 to 3) to prevent possible immune rejection of the transplanted cells. Immunoblot analysis was done by using 5 μl of the prepared homogenates.

RESULTS

Functional Studies of the Gene-Regulatory System in Transient Transfection Assays. To analyze the regulatory feature of the C-terminal deletion mutant hPRB891, we constructed a chimeric regulator (pGL) by fusing the ligand-binding domain of hPRB891 (residues 640–891), as a ligand-dependent regulatory domain, to the yeast transcriptional activator GAL4 (residues 1–94) (Fig. 1). This region of GAL4 contains a DNA-binding function (residues 1–65) (9), a dimerization function (residues 65–94) (10), and a nuclear localization signal (residues 1–29) (11). By replacing the DNA-binding domain of the progesterone receptor with that of GAL4, we eliminate the possibility of simultaneous activation of any endogenous progesterone-responsive genes. The reporter plasmid consists of the CAT gene driven by four copies of the consensus GAL4-binding site (17-mer) upstream of the *tk*

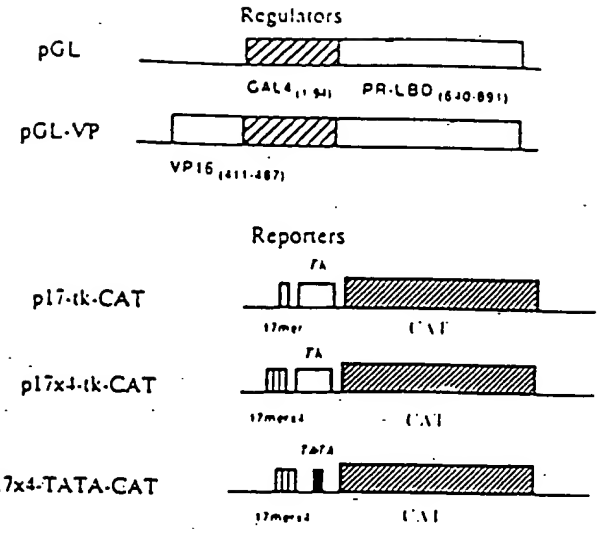

FIG. 1. Diagram of chimeric regulators and reporter constructs used in the regulatory system. The chimera GL consists of the ligand-binding domain (LBD) of the human progesterone receptor hPRB891 (residues 640–891), the DNA-binding domain of yeast-activator GAL4, and the activation domain of herpes simplex virus VP16. TK, thymidine kinase.

promoter (Fig. 1). The specific feature of this regulatory system is that it should only activate the target gene containing the GAL4-binding sites in the presence of RU 486. Because GAL4-activated genes are not known to exist in mammalian cells, this regulator should be specific for the target gene of interest.

In the initial tests of the functional properties of this regulatory system, we cotransfected the regulator and reporter plasmid into CV-1 cells. The chimeric regulator pGL was unable to activate the reporter gene in the presence of RU 486 (Fig. 2*A*). This finding suggests that this mutated progesterone receptor ligand-binding domain by itself has very low transactivational capacity under our experimental conditions. To enhance the transactivational potential, we fused the C-terminal fragment of VP16 (residues 411–487) to the N terminus of GAL4 in the chimera GL, creating another chimeric regulator pGL–VP (Fig. 1). This viral C terminus is acidic in nature and contains a potent transactivation function (12), which interacts directly with transcription factors TFIIB (13), TATA-binding protein (TBP) (14), and TBP-associated factor $TAFII_{40}$ (15) within the transcriptional preinitiation complex. Cotransfection of the regulator, pGL–VP (0.2 μg), with the reporter plasmid p17x4-tk-CAT (5 μg) into CV-1 cells resulted in a 7- to 10-fold induction of CAT enzymatic activity in the presence of RU 486 (Fig. 2*A*), whereas the potent progesterone agonist RU 27987 had no effect. As a control, GAL4-(1–94) showed no activation of the reporter plasmid, whereas GAL4–VP constitutively activated CAT expression. These results demonstrate that the chimeric regulator pGL–VP could activate the expression of a target gene only in the presence of the antiprogestin RU 486.

To assess the effect of promoter on reporter gene expression, we replaced the *tk* promoter with a minimal promoter containing only the TATA box sequence from the adenovirus *E1B* gene (7). After cotransfecting this reporter plasmid (p17x4-TATA-CAT) (5 μg) with pGL–VP (2 μg), we noticed that the basal activity of this reporter was significantly lower than that of p17x4-tk-CAT (5 μg) (Fig. 2*B*). When RU 486 was added, an ≈50-fold induction of CAT activity was observed, in contrast to only 10- to 15-fold induction using p17x4-tk-CAT. However, the level of maximal activity of p17x4-TATA-CAT was lower. This result demonstrates that other sequences

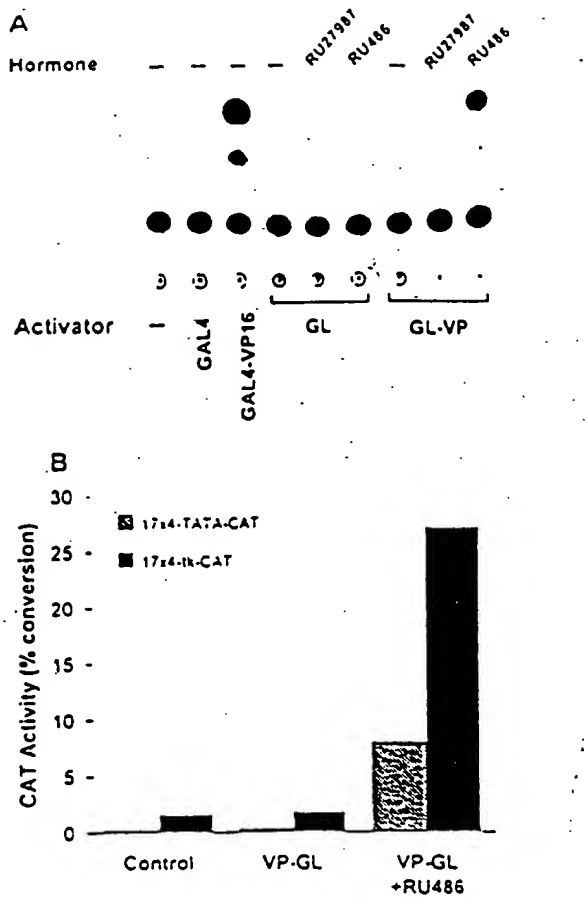

FIG. 2. Inducible expression of target gene by RU 486 and effects of different promoter use on transcriptional activation. (*A*) CAT assays showing cotransfection of the reporter plasmid (p17x4-tk-CAT) (5 μg) either alone (left lane) or with various GAL4 derivatives (0.2 μg) in CV-1 cells. Cells were incubated with RU 486 (10^{-7} M), RU 27987 (10^{-6} M), or solvent (80% ethanol) for 36 hr, as indicated. GAL4 consists of the DNA-binding domain (residues 1–94), whereas GAL4–VP16 is a chimeric protein consisting of GAL4 (residues 1–147) with VP16 (residues 411–490) fused at the C terminus. (*B*) CAT activity represents an average value of two cotransfection experiments with 2 μg of regulator and 5 μg of reporter plasmids, as indicated.

in the *tk* promoter such as GC and CAAT box sequences were likely contributing to the overall transcriptional activity observed. Our observation indicates that the reporter construct with the minimal TATA promoter could be used in situations where a low basal level of expression of the target gene is preferred, whereas the *tk* promoter construct could be used when higher overall activity is desired and a low basal activity can be tolerated. In addition, we showed that multiple copies of 17-mer are preferred to achieve maximal induction by this regulatory system (data not shown).

Dosage–Response of the Gene-Switch System to Progesterone Antagonists. To further characterize the chimeric regulator pGL–VP we established a stable transfected rat fibroblast cell line that expresses TH in response to RU 486. We chose TH as a target gene because it is a stable and well-characterized rate-limiting enzyme in the synthesis of dopamine, known to be deficient in striatum tissue in the brains of patients suffering from Parkinson disease (16). A permanent cell line (T1.21) was selected on the basis of its strong induction of TH by RU 486. We measured TH expression in response to various progesterone agonists and antagonists using immunoblot analysis (17). Fig. 3*A* shows that pGL–VP could activate TH expression in response to all progesterone

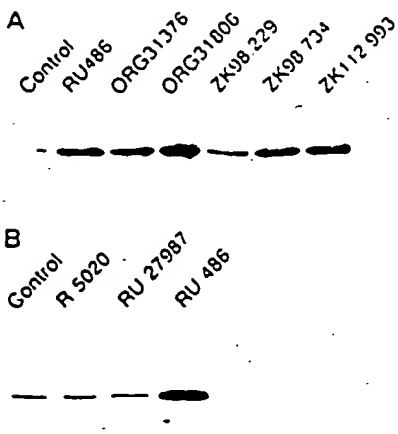

FIG. 3. Characterization of the effect of progesterone antagonists and agonists on the chimeric regulator in cell culture. (*A*) Chimeric regulator GL–VP responds to various progesterone antagonists. The stable fibroblast cells (T1.21) containing regulator plasmid pGL–VP(H) and target plasmid 17x4-tk-TH(N) were incubated with various progesterone antagonists, as indicated, at 10 nM for 24 hr. Levels of TH from cell extracts were analyzed by immunoblotting (17). (*B*) Activation of TH gene expression by regulator GL–VP is ligand-specific. Cells were incubated with R 5020, RU 27987, RU 486 at 10 nM. In control experiment, 80% ethanol (solvent used for steroids) was added to cells. Cell extracts were prepared and analyzed for TH by immunoblotting assays.

antagonists tested (RU 486, ORG 31376, ORG 31806, ZK 98.229, ZK 98.734, and ZK 112.993) at 10 nM concentration. The induction was ≈10- to 20-fold, as judged by densitometric scanning (data not shown). Most importantly, this pGL–VP regulatory function is specific for progesterone antagonists only, in that none of the progesterone agonists (R 5020, RU 27987, or progesterone) could induce TH gene expression (Fig. 3*B*).

It is well documented that for RU 486 to act as an antagonist of progesterone and glucocorticoid, its concentration must reach the micromolar range (18, 19). This fact is illustrated by the clinical use of RU 486 as an abortion pill, in which high doses of RU 486 (600 mg, or ≈10 mg/kg) are used in combination with prostaglandins. However, because RU 486 is used as an agonist in this regulatory system, it appeared likely that expression of the target gene would occur at a concentration lower than that required for antagonistic activity. To investigate this, we incubated T1.21 cells with various concentrations of RU 486 and observed activation of TH expression at a concentration as low as 0.1 nM. The induction reached maximal levels at a concentration of ≈1 nM (Fig. 4*A*). This range of concentration for activation is 1000-fold lower than that required for general endogenous antagonism and agrees with our earlier findings that RU 486 binds to hPRB891 with a K_d of ≈3 nM (1).

Although endometrial changes have been shown to occur within 12 hr of RU 486 administration (20), how long it would take for RU 486, acting as an agonist, to exert its effect on gene expression is unclear. For this purpose, we determined the time course for RU 486 induction of TH expression. We showed that TH expression was detected within 2 hr after RU 486 administration and reached maximum at ≈10 hr (Fig. 4*B*).

Validation of the Regulatory System *in Vivo*. To verify the regulatory system *in vivo*, we transplanted the rat fibroblast stable cell line (T1.21) into the rhomboid and trapezius muscles of Sprague–Dawley rats. Twenty-four hours after cell transplantation, RU 486 or control vehicle was given for 2 days at the indicated dose (Fig. 4*C*). Three days after implantation, muscles containing the transplanted fibroblast cells were ex-

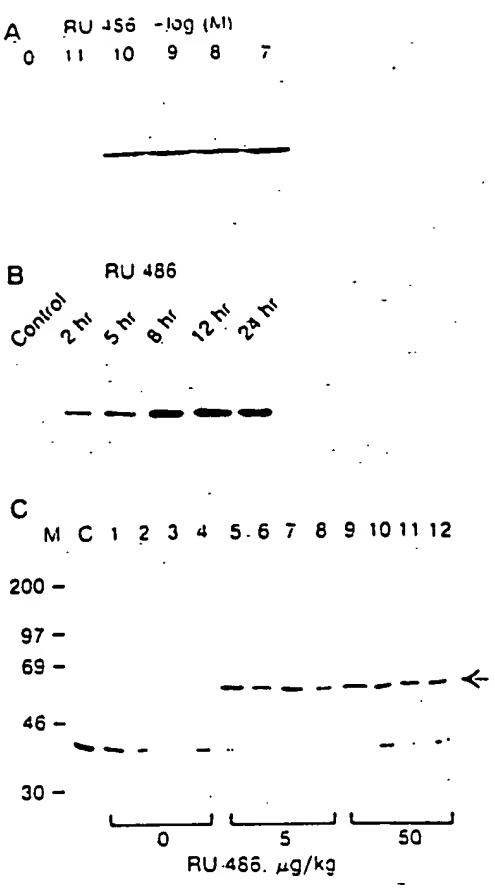

FIG. 4. Dose–response time course of RU 486-mediated activation of gene expression and characterization of the gene-switch system *in vivo* with transplanted cells. (*A*) Dose–response of RU 486 activation on TH expression. Cells (T1.21) were incubated with different concentrations of RU 486, as indicated, before harvesting for immunoblot analysis. Approximately 4×10^5 cells were seeded for each 60-mm culture dish; hormones were added the next day and incubated for another 24 hr. Solvent (80% ethanol) instead of RU 486 was added to the plates in the control experiment (labeled as 0). (*B*) Time course of RU 486-mediated TH expression. Cells (T1.21) were seeded onto 60-mm dishes at 4×10^5 cells per plate and incubated with RU 486 (100 nM) for different periods, as indicated, and harvested at the same time. (*C*) Immunoblot analysis of rat muscle extracts transplanted with fibroblast cells (T1.21). Lanes: 1–4, controlled rats received only sesame oil; 5–12, rats were given RU 486 injection at the indicated doses; C, extracts were prepared from nontransplanted muscle of a rat that had received RU 486. Arrow, TH levels.

cised and analyzed for TH expression. Fig. 4*C* shows that RU 486 induces expression of the TH gene at a dose of 5 μg/kg, and the expression levels are similar to those in rats that received 50 μg/kg. Similar results were obtained in identical repeat experiments when cells were transplanted into rats treated with or without cyclosporine and in transplants into nude mice (data not shown). Analyses of repeat experiments indicated a >10-fold induction of TH by RU 486 at a dose of either 5 μg/kg or 50 μg/kg. The extracted TH was proven biologically active, and an 8-fold induction by RU 486 was measured by its enzymatic activity (data not shown). From these transplantation experiments, we conclude that our regulatory system also works efficiently *in vivo* upon administration of exogenous ligand at an optimal therapeutic dose.

DISCUSSION

Several regulatable systems have been reported over the past decade, including gene induction by steroidal hormones (21, 22), isopropyl β-D-thiogalactoside (23), tetracycline (as repressor) (24), or heavy metals (25). While each system has its own merits for application in mammalian cell cultures and even transgenic animals, these chemicals (ligands) either have the potential to activate other endogenous genes (in the case of glucocorticoids and estrogens), are inefficient, or are too toxic (isopropyl β-D-thiogalactoside) (26) to be used in animal and human gene transfer. A cell-membrane regulatory system has been reported recently that uses a synthetic ligand (FK1012) made of a dimer of FK506, an immunosuppressive drug, to activate a cellular tyrosine kinase pathway (27). Although this approach has use for pathway regulation, the system cannot be coupled with a specific target gene because the membrane receptor-activated kinase cascade would be expected to interfere with the expression of multiple endogenous genes. We believe that the optimal route of target-gene manipulation is via transcriptional regulation because the rate of target-gene expression can be controlled directly. The regulatory system we have presented eliminates many of the shortcomings of the systems mentioned above.

In our gene-switch regulatory system, we used a consensus GAL4 DNA-binding site (17-mer) in the construction of the target reporter plasmid. Because GAL4 has not been found in mammalian cells and no mammalian target genes are known to be activated by GAL4, it is unlikely that the chimeric regulator will inadvertently activate the expression of any endogenous gene(s).

As compared to i.v. injections, it has been shown that the bioavailability for oral administration of RU 486 is ≈40% in rats and humans (28). The i.p. administration of 5 μg/kg would therefore be equivalent to an estimated oral dose of ≈10 μg/kg or 0.6 mg in humans, a dose equivalent to Premarin, an oral estrogen used currently for postmenopausal replacement in women. This means that RU 486 can activate gene expression in this regulatory system at a significantly lower (at least 1000-fold) dosage than what is used as a progesterone antagonist in inducing abortion (10 mg/kg). After oral administration of 200 mg of RU 486, its serum concentration has been demonstrated to be ≈1–5 μM (29). Thus, the dosage of RU 486 used in these transplantation experiments (5 μg/kg) would translate to a serum concentration of ≈1–6 nM (if extrapolation is linear), which is similar to the concentration required for activation in our cell culture studies (Fig. 4*A*). From these transplantation experiments, we conclude that our regulatory system also works efficiently *in vivo* upon administration of exogenous ligand at an optimal therapeutic dose.

It has been reported that side effects from the use of RU 486 generally have been moderate (mainly fatigue or mild rash), even in long-term administration of 200 mg/day orally (30). These side effects are mostly due to the antiglucocorticoid activity at this high dosage (31). Because RU 486 is used as an agonist in this gene-switch system, we believe that there will be no significant antiglucocorticoid or other deleterious effects at this low dosage in animals or in humans.

This prototype gene-switch system presented here and its subsequent modifications can have wide application to related research areas, such as experiments involving transgenic animals. By placing a tissue-specific promoter with the regulator, it should be possible to regulate both tissue-specific and inducible expression of a given target gene to study its function *in vivo* in transgenic animals. In addition, developmental studies in transgenic animals would be feasible because RU 486 has been shown not to be an abortifacient in rats at doses <0.5 μg/kg (32). Perhaps most importantly, its inducible characteristics can be used to generate disease models (or induce therapeutic proteins) in transgenic animals at adult periods of their life cycles. In this way, early developmental defects or even lethality could be prevented by using this inducible system because no expression of the target gene

would occur until RU 486 is administered to the animals at specific developmental or adult stages. Finally, the ability of RU 486 to distribute widely in the brain (29) allows this switch system to be used in the central nervous system.

In conclusion, our gene switch/regulatory system uses an existing drug that is orally effective and has an established safety record (33), thereby circumventing certain of the time-consuming and costly steps required to develop a new drug. This gene-switch system reported here represents a regulatory system that could be applicable for gene transfer to animals, as well as to humans, in which the delivered gene(s) can be specifically turned on/off in response to an exogenous compound. Future modifications of this regulatory system could include manipulations of the promoter sequence of the reporter and the DNA-binding and transcriptional activation domains of the regulator to yield regulatory switches with lower basal levels and/or higher inducibility.

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